

Finger Pointing: Engineered Zinc-Finger Proteins Allow Precise Modification and Regulation of Genes

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Gene therapy works, as several children cured of the severe immune disorder called “bubble boy disease” can attest. But as currently practiced, the method is imprecise—sometimes the new DNA lands in an unsuitable part of the genome, doing more harm than good. Fortunately, a recent advance in gene targeting may soon improve this outlook. This new technology is based on a class of natural DNA-binding molecules called zinc-finger proteins (ZFPs) that can be engineered to precisely target any part of the genome. When combined with a suitable effector domain, the ZFP can activate, suppress, mutate, or repair the selected gene. Al-

Xenopus frog. Since then, researchers have identified ZFPs in many other species, including humans—indeed, more than 700 human genes code for them. Klug and his colleagues demonstrated that each finger independently binds to a specific sequence of three base pairs (bp) of DNA that depends on the protein's amino acid composition. “It's a powerful and unique system for gene intervention,” says Klug.

Barbas, who had earlier studied DNA targeting using small molecules, became intrigued by this discovery. In the early 90s, he built ZFs that recognized all DNA triplets of the form *Gnn*. His team at

replacement gene sandwiched between sequences identical to those flanking the original gene, in the hope that the cell's repair machinery would swap the old DNA for the new. Unfortunately, such “homologous” recombination happens only in about one in a million cells: a high enough rate for making knockout mice but too low for manipulating other organisms, including rats. One way to increase it is to introduce a double-stranded break in the DNA on or near the gene. But this is easier said than done—DNA can be cut with endonucleases, but doing this at the right spot on the genome is tricky. “So I looked around for reagents that could make targetable double strand breaks,” says Carroll.

In 1996, Carroll found what he was looking for. A group at Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, led by Srinivasan Chandrasegaran, Ph.D., had just built the first ZF nuclease, combining a ZFP with the cleavage domain of the *FokI* bacterial nuclease and successfully marrying the specificity of the one with the DNA-snipping capability of the other. “I got really excited about its potential to target recombinogenic double-strand breaks,” says Carroll. However, the first ZF nuclease Carroll obtained from Chandrasegaran didn't hit the expected target. In solving this puzzle, the researchers discovered that the *FokI* domain has to dimerize before it can act; cleavage occurs when two matching ZF half-nucleases bind DNA head-to-head, effectively assembling the cleavage reagent at the target site. Since both molecules have to recognize their targets, the double binding ensures high specificity. “This is one of the absolutely fabulous features of these reagents,” says Carroll. “It would have been very difficult to design, but we got it for free.”

After showing that ZF nucleases could make double-stranded breaks and trigger homologous recombination in *Xenopus* egg cells, Carroll turned his attention to *Drosophila*. Armed with a set of ZFs from

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though zinc-finger (ZF) technology is still in development, promising clinical results are already emerging. “What we hope to achieve during the next few years is the ability to put a gene at any given place in the genome, and to fix or cut out any specific disease-causing gene,” says Carlos Barbas, III, Ph.D., of The Scripps Research Institute (TSRI) in La Jolla, California. “With this technology, we can shape the genome in ways that would otherwise be impossible.”

Naturally occurring ZFPs are transcription factors (TFs) that home in on and bind to specific DNA sequences. Modular in structure, the molecules grip the DNA using a number of elongated units, each made of about 30 amino acids folded into a compact domain and stabilized by zinc ion. “I deduced the existence of this repeated motif, which I called zinc finger (ZF), and realized it would be a powerful method for making general DNA-binding proteins,” says Sir Aaron Klug, a Chemistry Nobel Laureate who discovered the proteins in 1985 in the egg cells of the

TSRI has since added most of the *Cnn*, *Tnn*, and *Ann* triplets to their library. The researchers showed that two or more fingers could be placed in tandem to recognize longer sequences, and the ensemble could be coupled to various effector domains such as transcription activators or repressors. In 2000, Barbas targeted on the first human gene using this technology—the *erbB2* oncogene implicated in breast tumors. This was a challenging target: the 18bp stretch in *erbB2* the ZFP was designed to recognize differed by only 3 bp from an *erbB3* segment. Despite this potential for confusion, the protein hit its mark accurately; a similar molecule that targeted *erbB3* was equally precise. “That told us that we could reach into the genome and specifically tickle any given gene,” says Barbas.

Meanwhile, Dana Carroll, Ph.D., at the University of Utah in Salt Lake City, Utah was looking for a more efficient way to target genes. The existing method, widely used even now, was to insert into the nucleus a strand of donor DNA carrying the

the Barbas lab, his team successfully knocked out the fruit fly's *y* gene. "It showed for the first time that we could hit a real gene at its normal genomic site in a real organism," says Carroll. Since then, Carroll and other researchers have obtained similar results with a number of other organisms, including the zebrafish, the roundworm, and the tobacco plant, as well as with several mammalian cell types, including human cancer and stem cells. They have shown that even a transiently expressed nuclease can induce a permanent and heritable change in the cell's genome.

In the 90s, two companies sprang up around this new technology: UK-based Gendaq, founded by Klug, and Richmond, California-based Sangamo Biosciences. Sangamo, now a publicly traded company with a market capitalization of about \$100 million, acquired Gendaq in 2001. In 2002, Sangamo researchers demonstrated regulation of the *VEGF-A* gene in mice. "It was the first successful use of a ZF TF in a human disease model," says Philip Gregory, D. Phil., vice president of research at the company. This compound is now in clinical trials for diabetic neuropathy and Lou Gehrig's disease. In 2003, researchers engineered a six-finger protein that inhibited the checkpoint kinase 2 (*CHK2*) gene while leaving all 16,000 other genes in two cell lines unaffected. "We demonstrated the astonishing specificity that's possible with these reagents," says Gregory. This year, Sangamo researchers successfully made a human T cell line resistant to HIV by knocking out the *CCR5* gene with a nuclease. The company expects to file an investigational new drug (IND) application this year and to conduct a phase I trial of this compound.

Precise gene targeting potentially eliminates the risks associated with random transgene insertion, which is a major concern with conventional gene therapy. But

ZF technology offers other potential benefits as well. Unlike transgenes, engineered TFs act "naturally" on endogenous genes, ensuring that all splice isoforms of the target proteins are produced in the correct ratio. For instance, tissues given a *VEGF-A* transgene or the growth factor itself grow leaky vessels due to missing isoforms. In contrast, mice treated with a ZF TF in the Sangamo *VEGF-A* study developed normal blood vessels. ZF therapy is particularly suitable for conditions such as cancer that are more often related to dysregulated gene expression rather than mutations. "We can use these proteins to reprogram a cancer cell to become more epithelial-looking by reactivating tumor suppressor pathways," says Pilar Blancafort, Ph.D., of the University of North Carolina at Chapel Hill.

From the practical standpoint, it is easier to work with a small gene therapy vector carrying a TF rather than a typically larger one encoding cDNA. This also makes targeting multiple genes easier. "We can hit the cell several times, one after the other, and whatever epigenetic change we induce will stay and reinforce one another," says Marianne Rots, Ph.D., of the University Medical Center Groningen in the Netherlands. Correcting mutations is also simpler with ZF technology—instead of a complete replacement gene, only a small segment with the correct sequence needs to be provided.

Promising as it sounds, though, ZF technology has to surmount many challenges before it is ready for the clinic. The ZFP's specificity, its chief attraction, is often imperfect; even six-finger proteins sometimes bind incorrectly. This is less of a concern for TFs, since a wayward molecule will rarely hit a coding region. For nucleases, however, the problem is serious—an off-target DNA break could kill the cell. To prevent incorrect binding, researchers such as David Segal, Ph.D., of

the University of California at Davis, have reengineered the *FokI* dimerization interface. "We took a nuclease that was toxic to cells and turned into something that wasn't toxic," says Segal.

An engineered ZFP is typically tested first on "naked" DNA in vitro. To make it work inside the cell, however, the nature of the chromatin at the gene locus, how the DNA wraps around the nucleosome, and other epigenetic factors have to be considered. "All this will have an impact on how the ZFPs will work, but people have not looked much into that," says Pernette Verschure, Ph.D., of the University of Amsterdam in the Netherlands. Fortunately, studies indicate that ZFPs are not greatly impeded by some epigenetic barriers such as methylated or condensed chromatin. "We are able to reach DNA targets that we thought were completely inaccessible," says Blancafort.

Despite its modular structure, assembling a working ZFP is trickier than simply connecting a bunch of ZFs in tandem. Not all permutations of ZFs are compatible; finding ones that work often involves complex design strategies as well as considerable trial-and-error. Researchers unwilling to invest this much effort could instead purchase custom-made ZF nucleases from Sigma-Aldrich, which started marketing the compounds under license from Sangamo this September. Currently, the price tag is high—about \$25,000 per nuclease kit, according to Sigma-Aldrich market segment manager Phil Simmons—but will eventually decrease once the reagents become off-the-shelf commodities. "Once people can go online to order their ZFPs, it will be the technology of choice to modify the genome," says Barbas.

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